Application of mass spectrometry to hair analysis for forensic toxicological investigations

APPLICATION OF MASS SPECTROMETRY TO HAIR ANALYSIS FOR FORENSIC TOXICOLOGICAL INVESTIGATIONS

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ABSTRACT

The increasing role of hair analysis in forensic toxicological investigations principally owes to recent improvements of mass spectrometric instrumentation. Research achievements during the last six years in this distinctive application area of analytical toxicology are reviewed. The earlier state of the art of hair analysis was comprehensively covered by a dedicated book (Kintz, 2007a), that represents key reference of the present overview. Whereas the traditional organization of analytical methods in forensic toxicology divided target substances into quite homogeneous groups of drugs, with similar structures and chemical properties, the current approach often takes advantage of the rapid expansion of multiclass and multiresidue analytical procedures; the latter is made possible by the fast operation and extreme sensitivity of modern mass spectrometers. This change in the strategy of toxicological analysis is reflected in the presentation of the recent literature material, which is mostly based on a fit-for-purpose logic. Thus, general screening of unknown substances is applied in diverse forensic contexts than drugs of abuse testing, and different instrumentation (triple quadrupoles, time-of-flight analyzers, linear and orbital traps) is utilized to optimally cope with the scope. Other key issues of modern toxicology, such as cost reduction and high sample throughput, are discussed with reference to procedural and instrumental alternatives.

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I. INTRODUCTION

The analysis of biological specimens to detect of various types of drugs is consistently requested within forensic investigations as a necessary step to prove exposure to these substances. Although blood and urine remain the matrices of choice to detect recent exposure, hair analysis is gaining ever-increasing importance, as its potentials, limitations, and new application areas are progressively uncovered. Although, for most drugs, any evidence of exposure is erased from blood and urine a few days (or hours) after their intake because of the natural metabolic and excretion body processes, a minute portion of the same drugs and metabolites is ultimately incorporated through several routes (blood capillaries, sweat, and sebum) into the keratin structure of the hair, from which it is hardly removed. This drug-fixing into the hair structure resists hair growth for several months and leads to a potential chronological trace of drug exposure, with farther periods corresponding to the hair segments more distant from the hair root. The continuous improvement of analytical procedures and instrumental technologies allows one to determine the very small amounts of drugs included into the hair at ever-decreasing concentrations, insomuch as there are currently several scientific reporting of drug detection in hair after a single exposure. Progresses in chromatographic and mass spectrometric techniques have both equally contributed to the impressive results achievable nowadays with modern forensic toxicology in hair analysis.

On the negative side of hair analysis, there are several potential sources of bias that should always be taken into account in order to produce reliable conclusions from experimental results. First of all, hair is not a homogeneous matrix; thus, careful and representative hair sampling is a necessary prerequisite of correct analysis. Secondly, the degree of hair incorporation depends on the chemical structure and related properties of the drug, including melanin affinity, lipophilicity and membrane permeability (Kintz, 2012a). Third, adsorption of external substances from the environment is occasionally claimed as a source of false-positive results, especially for professionally-exposed subjects and volatile substances, such as, for example, cocaine (LeBeau & Montgomery, 2009). To prevent this inconvenience, preliminary washing of hair samples is always recommended to eliminate any possible interfering substances adsorbed onto the external surface of the hair. Segmental analysis and acceptance of cut-off values often rule out the chance that exogenous drug sources might have contaminated the internal keratin structure. Forth, strong cosmetic hair treatments, such as the use of oxidants or highly basic coloring, might damage the keratin structure to favor release of incorporated substances to finally lead to false-negative results. Also, repeated shampooing might partly wash out the hydrophilic drugs incorporated into the hair.

Lastly, the use of cosmetic products such as grease, sprays, and gels might also interfere with hair analysis.

A comprehensive review of drug testing in hair was published in a dedicated book edited by Pascal Kintz (2007a). Since then, only one review was published on abused drug analysis in hair samples, in which chapters are organized on the basis of the various classes of substances (Wada et al., 2010). The present review updates Kintz's book, under the perspective of its chapters 10-15 (Kintz, 2007a); namely, under application areas point of view. The studies published before 2006 are only incidentally cited in the present review, whereas extensive coverage of the papers published in the last six years is presented.

II. APPLICATION AREAS OF MASS SPECTROMETRY TO HAIR ANALYSIS IN FORENSIC TOXICOLOGY

A. Targeted Drug Screening for Specific Drug Classes

Although screening of psychoactive substances in biological specimens might have a variety of objectives, in terms of target chemical classes and purposes of control, legislation of most countries make a clear distinction between drugs of abuse, whose use is prohibited in almost all circumstances, and pharmaceutical substances, whose use is permitted under medical control and prescription, even though they are occasionally abused. Most routine screening, such as, for example, workplace testing, are addressed to the drugs of abuse only, whereas other psychoactive pharmaceuticals are searched in selected subjects and follow anamnestic indications. In particular, detection of the most-common drugs of abuse in hair samples (Kintz, 2007a; Kintz et al., 2006; Pragst & Balikova, 2006; Wada et al., 2010) is increasingly requested for the retrospective withdrawal control of habitual drug abusers, as well as in other toxicological investigations, such as workplace drug testing [EWDTS (European Workplace Drug Testing Society), 2010; Caplan & Goldberger, 2001], driving re-licensing, occasional or pre-natal exposure to drugs (Kintz, 2007b; Falcon et al., 2012), and post-mortem toxicology. Therefore, although hair samples are routinely collected and analyzed in forensic and toxicological laboratories, most published methods were implemented to determine class-specific groups of compounds, mainly cannabinoids, amphetamines, cocaine, and opiates, even though the analytical procedures have been constantly updated (Kintz, 2007a; Pragst & Balikova, 2006; Wada et al., 2010).

1. Classes of drugs

Although the widespread abuse of hashish or marijuana makes the detection of long-term exposure to Δ^9-tetrahydrocannabinol (THC) by hair analysis extremely important in clinical and forensic contexts, drug inhalation and adsorption from environmental smoke are frequently claimed to justify positive detection of THC and/or its main metabolite. Although a cut-off of 0.1 ng/mg was traditionally recommended, more recently a cut-off of 0.05 ng/mg has been proposed as a suitable level to detect also occasional users (Pragst & Nadulski, 2005) and for confirmation analyses (Cooper et al., 2012).

GC/MS is the technique of choice for THC detection in the majority of published methods, and no particular analytical improvement has been proposed recently, except for the increasing role played by headspace solid-phase microextraction (HS-SPME) procedures. Nadulski and Pragst developed, validated, and routinely applied to driving-ability examination a new method for cannabidiol (CBD) and cannabinol (CBN) with improved sensitivity (Nadulski & Pragst, 2007). The authors used alkaline digestion of 15-30 mg hair aliquots, subsequent liquid-liquid extraction (LLE), automated HS-SPME after in-sample silylation, and GC/MS-SIM (selected ion monitoring) analysis. For THC, a limit of detection (LOD) of 0.012 ng/mg was obtained. A similar procedure without derivatization with 10 mg of hair sample was proposed (Rodrigues de Oliveira et al., 2007), but the reported LOD and LOQ (limit of quantification) values of 0.07 ng/mg and 0.12 ng/mg, respectively, are above the recommended cut-off limits and make the method apparently not appropriate for routine analysis. Emídio et al. developed and validated a method for the determination of THC, CBD, and CBN in hair samples, with HS-SPME combined with GC/MS/MS (ion trap) detection (Emídio et al., 2010). From a 10 mg hair sample, an LOD of 0.031 ng/mg and LOQ of 0.062 ng/mg were obtained; the latter was slightly higher than the cut-off value of 0.05 ng/mg. Nonetheless, from 10 hair samples from cannabis users, an average concentration of 0.056 ng/mg was found; i.e., below the reported LOQ.

Unlike THC, the improvement of existing analytical methods for stimulants, including cocaine and amphetamines, in hair samples has been widely investigated recently. Lee et al. used a standard GC/MS procedure, after derivatization with trifluoroacetic anhydride, in order to study the abundance ratio of methamphetamine (MA) and its metabolite amphetamine (AP) in hair and hence aid the positive results interpretation (Lee et al., 2009). High MA concentrations, together with low AP percentage, were related to severe and chronic drug abuse (Lee et al., 2009). In order to facilitate MA and AP analysis in hair, Miyaguchi et al. developed a simple and fast (1 hour) method for sample preparation named MiAMi (micropulverized extraction-acqueous acetylation-

microextraction by packed sorbent) followed by GC/MS (Miyaguchi et al., 2009). The amount of hair sample required for qualitative analysis based on full-scan mass spectra was only 5 mg, whereas 1 mg of a hair specimen was sufficient for amphetamine quantitation. The same group (Miyaguchi et al., 2007) previously published an extraction method on micropulverized hair for the HPLC-MS/MS determination of MA and AP, in which only 2 mg of sample were used.

An LC-MS/MS method that used electrospray ionization (ESI) and a triple quadrupole instrument was developed and validated by Chèze et al. to determine AP, MA, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA, "ecstasy"), 3,4-methylenedioxyethamphetamine (MDEA), and N-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine (MBDB) at low concentration levels, in hair, blood, and urine (Chèze et al., 2007). With 20 mg of decontaminated hair, the experimental LODs ranged from 0.3 pg/mg for MBDB to 6.3 pg/mg for MDA, where the recommended cut-off limit for amphetamines is 200 pg/mg (Cooper et al., 2012). The same study reported a real forensic case, in which the high sensitivity of the LC-ESI-MS/MS method was exploited to detect MDMA in a hair sample, after a single oral dose administration to an unaware victim (Chèze et al., 2007). Another recent study reported the development of an LC-MS/MS method for the simultaneous determination of a large set of amphetamine-like anorectics and their metabolites in hair samples (Lee et. al., 2012). The procedure was used to analyze the diffusion of anorectics abuse in Korea. The hair incorporation of MA and AP following controlled administration of MA to seven volunteers was recently studied (Polettini et al., 2012). For these amphetamines, significant dependence (i.e.; linear direct correlation) of drug incorporation on hair melanin content was clearly demonstrated.

Besides MALDI-imaging techniques, several traditional methods were recently proposed to determine the most common stimulant drug (i.e., cocaine) in hair samples. The suggested cut-off concentration is 500 pg/mg for cocaine and 50 pg/mg for its main metabolite, benzoylecgonine (BZE) (Cooper et al., 2012). A GC/MS analytical method to quantify cocaine and its main metabolite BZE in hair samples was proposed (Barroso et al., 2008). Despite the simple instrumentation utilized, the method proved to be sensitive and specific, and allowed one to detect 20 and 15 pg/mg of cocaine and BZE, respectively, from only 20 mg of sample.

Improved sensitivities were obtained with LC-MS/MS methods. Moore et al. developed and validated an LC-MS/MS procedure for the analysis of cocaine and its metabolites (BZE, cocaethylene, and norcocaine) in hair with an atmospheric pressure chemical ionization (APCI) source and a triple quadrupole (QqQ) mass analyzer (Moore et al., 2007). For all analytes, the LOQs (50 pg/mg) and LODs (25 pg/mg) made the method suitable for routine forensic analysis.

A simple and fully validated procedure for the qualitative and quantitative determination of opiates in hair was presented (Barroso et al., 2010a). This paper describes a GC/MS method for the analysis of codeine, morphine, 6-monoacetylmorphine (6-MAM), 6-acetylcodeine, and tramadol in 20 mg hair samples. The presence of the screened analytes was demonstrated in several real cases, among which it was proven that tramadol is occasionally abused by opiate addicts. A combination of opiates, cocaine and metabolites was simultaneously screened with an LC-ESI-MS/MS method (Huang et al., 2009). This method was fully validated and used in the analysis of 79 authentic hair samples, and demonstrated that a multiresidue approach might screen different classes of drugs at the same time.

The objective of cost reduction of workplace drug screening with a multiresidue UHPLC-MS/MS strategy was explicitly cited in a recent study (Di Corcia et al., 2012). Thirteen analytes, including opiates, cocaine, amphetamines, THC, buprenorphine, methadone, and a few metabolites, were simultaneously screened in hair samples. A simple sample preparation combined with multiclass analysis and fast chromatographic separation allowed one to obtain high sample throughput, together with excellent sensitivity and selectivity; the procedure is valuable for large sample workload and reduced costs of analysis (Di Corcia et al., 2012).

2. New instrumental set-up

A new and original study, aimed to the direct detection of MA in hair samples, used imaging mass spectrometry (IMS) to perform micro-segmental analysis (Miki et al., 2011). In practice, a hair shaft was affixed to a carbon tape and manually cut lengthwise to produce micro-incisions at extremely close range with a razor and a microscope. After matrix deposition, IMS was obtained by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) and MALDI-Fourier transform ion cyclotron resonance (FTICR) mass spectrometers. IMS produced a barcode-like image of methamphetamine on the longitudinal sections of the hair shafts, obtained from a methamphetamine chronic abuser, to indicate the periods and sequence of single MA intake (Miki et al., 2011). This innovative approach appears to be very promising in the forensic context, as soon as the hair sample preparation can be automated.

A MALDI hybrid triple quadrupole linear ion trap (QqQ_{LIT}) instrument, equipped with a high repetition rate laser, was recently proposed to perform MS imaging on a single hair, for cocaine consumption monitoring (Porta et al., 2011). The QqQ_{LIT} technology was exploited to simultaneously achieve target quantification with selected reaction monitoring (SRM) mode of operation and sensitive MS^3 acquisition for confirmatory analysis. MS imaging of intact single hair

sample offers much higher resolution than segmental LC-MS/MS analysis, and only depends on the distance between two adjacent laser shots. However, the minimal 0.1 mm resolution allowed by the laser and corresponding to a theoretical average hair growth of less than 12 h, has currently little significance in forensic investigations, beacuse the complex nature of keratin incorporation mechanisms and the heterogeneous physiology of hair growth might provide a much larger bias on chronological assessment. Single washed hair samples were fixed onto a stainless steel MALDI plate with a double-sided adhesive foil, and the MALDI matrix was manually sprayed. First, an imaging profile of cocaine and metabolites was acquired in the SRM mode over the whole hair length, taking into account that highly selective transitions should be chosen to compensate for the absence of chromatographic separation. Secondly, MS/MS and MS^3 experiments were performed for confirmatory identification. The entire analytical workflow is represented in Figure 1 (Porta et al., 2011). An experimental LOD of 5 ng/mg allows for cocaine detection in the hair of chronic abusers, but not after a single administration. Moreover, scaling down to single-hair analysis makes the choice of representative sampling crucial, unless a further source of uncertainty and bias is introduced.

In order to detect all cocaine metabolites, including the highly polar ecgonine methyl ester (EME), Quintela et al. developed and validated a hydrophilic interaction liquid chromatography (HILIC) method to be coupled to tandem mass spectrometry (Quintela et al., 2010). With as little as 10 mg of specimen, experimental LODs were better than or equal to 1 pg/mg. The recognition of ultra-trace amounts, at low pg/mg of hair, appears to be crucial for EME detection, because this metabolite has a very low incorporation rate into the hair shaft (Quintela et al., 2010). Recently, Thibert et al. adopted a clean-up process for hair extracts based on molecularly imprinted polymers (MIP) selective for cocaine and BZE, followed by LC-MS/MS analysis (Thibert et al., 2012). An LOD lower than 70 pg/mg was reached for both molecules, slightly above the suggested cut-off value for BZE (Cooper et al., 2012).

A fast, but only qualitative, screening method to detect cocaine and its metabolites from hair samples used MALDI-TOF technology (Vogliardi et al., 2009). The whole hair sample preparation for MALDI analysis turned out to be significantly more rapid and simpler than for GC/MS, and data acquisition in MALDI analysis is, in turn, much quicker than in GC/MS. Thus, the MALDI technique shows clear advantages over the traditional approach, when fast screening of a large number of sample is required, as the same authors subsequently demonstrated by validating and testing the new method on 304 real hair samples (Vogliardi et al., 2010). High throughput and fit-for-purpose principles both find neat application in this well-designed example of fast cocaine screening in hair samples.

B. Non-Targeted Drug Screening

Most of the methods cited in the preceding chapter are specific for a class of drugs. The highly-focused objective of these analytical investigations allows one to optimize sample treatment, clean-up, and chromatographic conditions in order to maximize recoveries and to meet the acceptance criteria of sensitivity and specificity that lead to accurate quantification for confirmation. However, their applicability in general drug screening for acute intoxication and autopsic analysis appears limited. In practice, these class-specific procedures find useful application when the target analytes can be predicted in advance; for example, in heroin overdose fatalities.

In the daily activity of forensic laboratories, it is quite frequent that the target analytes cannot be foreseen, so that a wide range or "general" screening of abused drugs is commonly required, as it occurs in most acute intoxications and post-mortem investigations. Whenever a multitude of candidate substances might represent the cause of intoxication or death, several analytical procedures are likely to be used on the collected biological specimen, with a direct impact on time, costs, and efficiency. For this reason, comprehensive screening procedures of multiclass drugs are progressively introduced into the analytical practice, even if the method conditions should compromise on absolute performances, in terms of recovery, sensitivity, selectivity, and accuracy, due to the presence of target analytes with widely different physico-chemical properties.

Even though hair is not the preferred biological matrix to ascertain acute intoxication, hair analysis is frequently commissioned to complete the circumstantial evidences; i.e., to ascertain whether the victim formerly abused drugs or whether any drug was administered to him/her earlier. To meet the high demand for drug screening in hair samples, toxicology laboratories are forced to update their procedures in order to target an ever-increasing number of drugs, but also to achieve rapid, simple, and sensitive testing with reduced sample preparation and fast instrumental processing so as to increase the overall sample throughput.

1. Methods based on GC separation

Cordero and Paterson developed and validated a GC/MS protocol to simultaneously quantify fourteen compounds, including some amphetamines, opiates, cocaine and metabolites, diazepam, and metabolite (Cordero & Paterson, 2007). A two-step derivatization with *N*-methyl-bis trifluoroacetamide (MBTFA) and *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) + 1% trimethylchlorosilane (TMCS) was used. Due to the general availability of GC/MS instrumentation in toxicology laboratories and the good analytical performances obtained in this study, this method

might find useful application in a variety of clinical and forensic investigations. An interesting evolution of the preceding method was developed by Aleksa et al., that used an HS-SPME-GC/MS to improve the protocol sensitivity (Aleksa et al., 2012). Seventeen drugs of abuse, including opiates, cocaine, amphetamines, and opioids, were reacted with the same derivatizing agents described above, and were detected at LOD levels of 130-200 pg/mg. The method was validated with only 5-10 mg of hair, and was especially useful for newborn, when sample availability is limited. Among the wide screening protocols to detect common panels of abused drugs, only few comprehensive methods include THC, regardless of the instrumental technique used. Merola et al. developed a new procedure with HS-SPME and GC/MS to provide concomitant determination of several drugs of abuse, including THC, but not opiates (Merola et al., 2010). A different approach and derivatizing agent, namely heptafluorobutyric anhydride (HFBA), was used (Wu et al., 2008a) to develop a GC/MS method for the simultaneous determination of amphetamines, opiates, ketamine, and their metabolites at LODs of 30-80 pg/mg. The same group (Wu et al., 2008b) improved selectivity and sensitivity to reach the low pg/mg level with electron capture negative chemical ionization (NCI) to detect the same derivatives.

TOF mass analyzers have also been used for broad drug screening in hair samples that take advantage of their full-scan and high-resolution capabilities. In an original study, Guthery et al. developed a GC×GC-TOF-MS procedure to provide a comprehensive qualitative drug screening of hair samples (Guthery et al., 2010). The hair extracts were subjected to derivatization with N-methyl-N-(tert-butyldimethyl)trifluoroacetamide (MTBSTFA), which efficiently reacted with a broad range of multiclass analytes, including opiates, opioids, cocainics, and benzodiazepines. The 2D chromatographic plot obtained from GC×GC separation offers an extremely clear depiction of the drug content profile, as Figure 2 demonstrates (Guthery et al., 2010).

2. Methods based on LC separation

Although the latter GC/MS methods proved suitable to test simultaneously several classes of drugs of abuse, the need to further expand the assortment of target substances within the same protocol, without the necessity of derivatization, made the highly flexible LC-MS/MS approach to multiclass screening more profitable and more widely used than GC/MS. An LC-APCI-MS/MS method was developed, validated, and applied for the retrospective multi-parameter evaluation and distribution of eleven drugs of abuse in hair samples (Klis et al., 2007). Subsequently, ESI was more extensively utilized than APCI, as in a validated LC-MS/MS method (Miller et al., 2008) that was aimed at the simultaneous quantification of amphetamines, opiates, cocaine and metabolites,

and diazepam and metabolites (17 compounds) in post-mortem hair samples. The use of an ion trap mass spectrometer was suggested (Bucelli et al., 2009) to screen 16 drugs of abuse in human hair. Despite the flexibility of ion traps to provide an easy switch between full-scan and MS/MS or MS^n modes of data acquisition, the method was not expected to provide accurate quantitative determinations, and needs further validation and a more extended panel before it might find widespread application in forensic laboratories. Di Corcia et al. proposed a simple sample extraction and direct injection into a UHPLC-MS/MS system to avoid solid-phase and liquid-liquid extraction and to achieve fast and simultaneous detection of the most common drugs of abuse, including THC, in hair samples (Di Corcia et al., 2012) . Lendoiro et al. developed and validated an LC-MS/MS multiclass screening method for the simultaneous detection of 35 substances, including THC, and the other most common drugs of abuse (opiates, amphetamines, cocaine, LSD, ketamine and scopolamine) and pharmaceuticals (benzodiazepines, antidepressants and hypnotics) (Lendoiro et al., 2012). For the entire range of investigated molecules, LODs were 0.2-50 pg/mg and achieved better cut-offs than Society of Hair Testing (SOHT) recommendations. In the screening mode of operation, only one SRM transition per compound was used, but the same procedure could be transformed into a confirmation method with addition of further target-specific SRM transitions (Lendoiro et al., 2012). Confirmatory analysis of ketamine and norketamine was the objective of a recent study that used molecularly imprinted solid-phase microextraction (MISPE) followed by LC-MS/MS (Harun et al., 2010).

An effective method to check for the presence of a wide set of drugs in hair samples was proposed (Nielsen et al., 2010), to validate an analytical method for the simultaneous screening and quantification of 52 common drugs and pharmaceuticals in hair with the novel UHPLC-TOF-MS technology. The panel of compounds was very broad because it included common drugs of abuse, benzodiazepines, analgesics, antidepressants and antipsychotics. The authors underlined, however, that the TOF instrument was less useful than a triple quadrupole on low-mass molecules, such as amphetamines, due to the presence of high background noise and the consequent lack of sensitivity and linearity (Nielsen et al., 2010). Similar broad drug screening, based on accurate mass measurements with LC-TOF-MS, was also achieved (Pelander et al., 2008). Also in this study, the potential to conduct a comprehensive screen (35 targeted analytes) was clearly demonstrated; however, the authors recommended more-specific instrumentation for target confirmation analyses. More recently, an automated screening method for the simultaneous identification and quantitation of 30 compounds (including most drugs of abuse, but also a few anabolic steroids, β-agonists and other pharmaceuticals) in hair samples was proposed with fast LC-TOF-MS (Domínguez-Romero et al., 2011). Progressive instrumental upgrading and method refinement provided improved

sensitivity with TOF mass spectrometers to allow the detection of the target compounds in the low pg/mg range (LODs ranged from 5 pg/mg to 50 pg/mg).

General screening of unknown drugs in hair samples was performed with LC combined with a quadrupole time-of-flight mass spectrometer (LC–QTOF-MS) (Broecker et al., 2012). Systematic collection of CID (collision-induced dissociation) spectra from quadrupole-selected protonated molecule used a data-dependent acquisition mode to create a wide dataset to allow the retrospective investigation of potentially toxic substances present in the hair sample. These CID spectra were compared with an accurate-mass CID spectral library created by the authors that contained more than 2500 toxicologically relevant substances (Broecker et al., 2011; Niessen, 2011). For 24 tested illegal drugs and benzodiazepines, significantly low LODs (3-15 pg/mg) were observed. The method was applied to hair samples from 90 death cases to reveal 212 substances altogether, even if only partial agreement with the positive identifications from urine and blood analysis was found, with many unexpected drugs detected and many reported drugs not detected in hair (Broecker et al., 2012). A similar approach was also applied to hair samples (Liu et al., 2010) with an in-house spectral library of about 800 substances for the identification of unknown drugs. In this case, the hybrid QqQ_{LIT} mass spectrometer produced low-resolution CID spectra from mass-selected precursor ions, with good sensitivity provided by the ion-accumulation capability of ion trap.

3. Other methods

Besides GC and LC, capillary electrophoretic methods were also occasionally used to separate drugs extracted from hair samples and to introduce them into the mass spectrometer. Capillary zone electrophoresis was combined with TOF analysis (CZE-TOF-MS) to detect four amphetamines, ephedrine, opiates, cocaine, and metabolites in hair samples. The method proved suitable for qualitative screening of all drugs below the prescribed cut-off limits, but was not fully satisfactory for accurate quantification, possibly due to the lack of deuterated internal standards (Gottardo et al., 2007; Gottardo et al., 2012). Also, another method, cation-selective exhaustive injection and sweeping micellar electrokinetic chromatography, proved compliant with the cut-off values in hair (Lin et al., 2007); however this method and the preceding study represented isolated proposals with no further application in common laboratory practice.

Quite uniquely, Jackson et al. proposed to eliminate the chromatographic separation with an ambient ionization technique, defined as low temperature plasma (LTP) ionization, to analyze biological specimens, including urine, saliva, and hair extracts (Jackson et al., 2010). The method was tested on 14 drugs that belonged to different classes (amphetamine, BZE, cannabidiol, caffeine,

cocaine, codeine, diazepam, ephedrine hydrochloride, heroin, ketamine, methadone, methamphetamine, morphine, and THC) with a linear ion trap mass spectrometer in the positive-ion mode. However, LOD values were rather high [ng/mL (i.e., 10 ng/mL) to the mg/mL concentrations] and only one real hair sample was analyzed that showed the presence of cocaine. The main focus of the study was on the potential of LTP ionization and its compatibility with miniaturized and portable instrumentation for on-site screening analysis.

C. Rape Drug Detection and Segmental Analysis

Drug-facilitated crime (DFC) is a general term that involves the administration of a drug to perpetrate a variety of crimes, including rape or other sexual assault, robbery, money extortion, as well as the deliberate maltreatment of elderly persons or children under the influence of psychotropic substances [UNODC (United Nations Office on Drug and Crime), 2011]. In most DFCs, the victims are subjected to violence or nonconsensual acts while they are incapacitated by the effects of a drug, often unknowingly ingested. Psychoactive substances used in DFCs might alter the victim's degree of consciousness, state of awareness, judgment, and memory. Such substances can make the victim vulnerable and unable to fight off the attacker, or are used to sedate the victim in order to facilitate his/her transport by the perpetrator. The drugs used for these criminal purposes include benzodiazepines, hypnotics, sedatives and anesthetics, drugs of abuse (such as cannabis, ecstasy, LSD, or heroin), miscellaneous drugs (e.g., scopolamine), and, most frequently, ethanol (Kintz, 2007b). The ideal substance to be dispensed to perpetrate a crime is the one that is readily available, easy to administer, able to rapidly impair consciousness, and produce anterograde amnesia (LeBeau & Montgomery, 2010).

Among DFCs, a drug-facilitated sexual assault (DFSA) occurs when a person is subjected to unwilling sexual acts while incapacitated or unconscious, due to the effects of ethanol, drugs, and/or other intoxicating substances (generally called "rape drugs"). It is highly unlikely that the specific drug used in a DFSA could be determined only by symptoms because the depressant effects of most of these drugs are similar. This factor makes crucial identification of the administered drug in the victim's biological specimen (Negrusz & Gaensslen, 2003). It is well-known that sexual assaults are significantly under- and late-reported. Evidently, any delay to report a DFSA implies that the victim's biological fluids (i.e., blood and urine) are not timely collected, hence rendering more difficult the toxicologists' task to identify the incapacitating agent (LeBeau & Mozayani, 2001). Whenever the natural metabolic processes have eliminated the drug from biological fluids (typically, urine and blood), hair is the most helpful specimen to be investigated (Negrusz et al., 2000; Villain et al., 2004; Negrusz et al., 2001; Scott, 2009; Parkin & Brailsford, 2009).

Toxicology laboratories are frequently asked to analyze hair from victims in order to reveal allegedly administered drugs. Further information is furnished by segmental analysis to generally provide chronological setting. Head hair should be collected at least four weeks after the alleged assault [UNODC (United Nations Office on Drug and Crime), 2011]. Since the average growth rate of human scalp hair is about 1 cm/month, administration of a single dose can be confirmed by drug detection in the corresponding segment, with no presence in the preceding and subsequent segments. The expected concentration in hair is generally in the low pg/mg range for most drugs (Kintz, 2007b; Villain et al., 2005). Therefore, the use of highly sensitive instrumental techniques is mandatory in the investigation of DFCs (Kintz et al., 2005; Chèze et al., 2005).

1. Methods based on LC-MS/MS with triple quadrupoles

The analytical strategy generally starts with a comprehensive screening for benzodiazepines and other drugs commonly involved in DFCs (Parkin & Brailsford, 2009). In recent years, several methods have been proposed for the simultaneous analysis of a large series of pharmaceuticals and drugs with LC-MS/MS with triple-quadrupole instruments. Hegstad et al. developed a multiclass LC-MS/MS method to screen several drugs of abuse on 20 mg of hair, which included 7-aminonitrazepam, 7-aminoclonazepam, 7-aminoflunitrazepam, oxazepam, diazepam, alprazolam, and two benzodiazepines-like z-drugs (zopiclone and zolpidem) (Hegstad et al., 2008). Similarly, Irving and Dickson described an LC-MS/MS procedure to detect sedatives in hair and nail samples (Irving & Dickson, 2007). Nine benzodiazepines, three metabolites, and zopiclone and its metabolite were selected and detected at low level in 50 mg specimen, with LODs that ranged from 0.02 pg/mg for zopiclone to 6.00 pg/mg for temazepam. This procedure was applied to the analysis of a hair sample collected 17 months after the occurrence of a DFSA: zopiclone and its metabolite were both detected in different hair segments at concentrations below 7 pg/mg (Irving and Dickson, 2007). Our group developed an LC-MS/MS method to detect 14 pharmaceuticals, including 10 benzodiazepines, ketamine, scopolamine, zopiclone, and zolpidem with LODs that ranged from 0.2 pg/mg for ketamine and zolpidem to 4.0 pg/mg for flunitrazepam (Salomone et al., 2012a). The method was applied to a real case of segmental analysis of the hair from two women, and showed conclusive discrimination between occasional and therapeutic administration (Salomone et al., 2012a). An interesting study of high-resolution segmental analysis on single hairs was developed to investigate the history of a multiple poisoning case with clozapine (Thieme & Sachs, 2012). From 1-2.5 mm segments of a single hair, a highly-specific LC-MS/MS procedure allowed to detect as little as 30 fg (1 pg/mg) of clozapine and its metabolite norclozapine. The collection of hair samples

after a 165 days interval, combined with the subsequent comparison of drug concentration profiles, provided an accurate estimation of hair growth rate for the selected individuals (Thieme & Sachs, 2012).

Another LC-MS/MS method was proposed and validated (Xiang et al., 2011) for the quantitative determination of 18 benzodiazepines in 20 mg of hair. LODs ranged from 0.2 pg/mg for estazolam to 5 pg/mg for 7-aminoclonazepam, 7-aminonitrazepam, α-hydroxytriazolam, and α-hydroxymidazolam. This method determined the distribution of estazolam after single dose intake in hair segments of healthy volunteers: estazolam concentrations ranged between 0.56 pg/mg and 2.60 pg/mg. Two real cases were studied with the same method, where the victims of DFCs were exposed to clonazepam: the presence of this drug was found in several hair segments from victim #1, and ranged from 1.63 pg/mg to 15.47 pg/mg, and in two segments from victim #2 at 1.31 pg/mg and 11.93 pg/mg concentrations. The metabolite 7-aminoclonazepam was found in hair segments at considerably higher concentrations than the parent drug to demonstrate the opportunity to include also the main metabolites in the benzodiazepine-screening procedure (Xiang et al., 2011). Similarly, Kim et al. established and validated an analytical method to simultaneously detect 27 benzodiazepines and metabolites plus zolpidem in hair with LC-MS/MS (Kim et al., 2011). The absolute LODs ranged from 0.005 ng (zolpidem) to 0.5 ng (bromazepam and chlordiazepoxide). The protocol was successfully applied to five forensic cases of either DFCs or benzodiazepines abuse, together with a study on drug incorporation into the hair of rats. As model for human hair, it was proved that rat-hair pigmentation does not have any significant effect on the degree of benzodiazepines (and metabolites) deposition in hair. As a matter of fact, the incorporation rates of other classes of drugs proved to depend on the hair pigmentation, to create ethnic discrimination (Gambelunghe et al., 2007; Polettini et al. 2012). The panel of screened compounds was expanded further by Lendoiro et al., who recently presented a target multi-analyte LC-ESI-MS/MS screening method to detect 35 licit and illicit drugs, including THC, in 50 mg hair samples (Lendoiro et al., 2012). Among the screened substances, zolpidem, zopiclone, and 12 benzodiazepines were detected at LODs that ranged from 2 pg/mg (7-aminoflunitrazepam, flunitrazepam, lorazepam, lormetazepam, nordiazepam, and diazepam) to 10 pg/mg (oxazepam, clonazepam, and tetrazepam). Analysis of seventeen hair specimens from various forensic cases demonstrated the method applicability: several illicit and prescription drugs were tentatively identified, and subsequently confirmed with two SRM transitions in place of one (Lendoiro et al., 2012). In all the cited LC-MS/MS procedures, triple-quadrupole instruments were used, and proved the efficiency of this instrument for wide multi-analyte screening. Similar LC-MS/MS configuration was also used in confirmation methods specifically designed to determine acepromazine (Gaulier et al., 2008),

diazepam, nordiazepam and tetrazepam (Laloup et al., 2007), sildenafil (Kintz et al., 2009), and gamma-hydroxybutyrate (Stout et al., 2010) in hair samples, with the purpose to investigate DFSA cases.

2. High-resolution mass spectrometers

Mass spectrometers other than triple quadrupoles were used for wide-range screening and confirmation analysis. Mass analyzers with high-resolution capability and accurate mass detection, such as TOF-MS and FTICR-MS, are particularly suited for *a-posteriori* reevaluation of the presence of drugs not considered in a preliminary target analysis. UHPLC-TOF-MS and UHPLC-MS/MS were both used in the development of a specifically validated confirmation protocol, aimed to quantification of triazolam in an emblematic case of DFC (Stybe Johansen and Dahl-Sørensen, 2012). In the hair segments (2 cm) that corresponded to the period of the alleged assaults, the presence of triazolam was revealed at a concentration of 1.0 pg/mg. A wider range of analytes was covered in a high-resolution mass spectrometric (HRMS) drug screening with TOF-MS (Pelander et al., 2008). The authors initially developed an analytical strategy for the comprehensive screening of drugs and doping agents in urine, but afterward the applicability of the method to other matrices, such as keratin, was also studied. Although the potential of the LC-TOF-MS approach to obtain a comprehensive drug screening in the hair of drug addicts was clearly proved, the study did not clarify whether the method sensitivity was high enough to detect drug traces from single intake. A UHPLC-TOF-MS screening procedure previously cited (Nielsen et al., 2010) targeted 52 analytes, among which 13 benzodiazepines and metabolites plus zaleplon, zolpidem, and zopiclone. For the screened molecules, homogeneous LODs were obtained that ranged from 10 pg/mg for diazepam and zolpidem, to 40 pg/mg for 7-aminoflunitrazepam and lorazepam. The method was applied to 15 autopsy hair samples. In most strands, several benzodiazepines were identified. Although accurate-mass analysis provided comparable selectivity to multiple SRM transitions from a triple-quadrupole instrument, the authors recommended positive findings to be confirmed with an additional method (Nielsen et al., 2010).

Orbitrap technology was also used for HRMS multi-target screening of hair samples (Vogliardi et al., 2011). The purpose of this study was to develop and validate a specific method based on LC-HRMS for the simultaneous detection in 50 mg hair strands of 28 benzodiazepines (and metabolites) registered in the Italian market. The "z-drugs" were not included, despite the fact that zolpidem is significantly prescribed among the Italian population. The use of a hybrid linear ion trap-Orbitrap mass spectrometer yielded LODs from 0.5 pg/mg (clotiazepam) to 10 pg/mg

(delorazepam). The validated procedure was applied to hair samples from suspected benzodiazepine consumers and showed its suitability for screening and identification of the target compounds. Indeed, the potential of such an instrument includes full-scan exact-mass measurement of protonated molecule ions and CID fragment ions, isotopic pattern recognition, and ion ratio determination between molecular and fragment ions. Recently, the same group developed and validated an analogous method to detect 28 substances that belonged to several drug classes: cocaine, amphetamines, opioids, benzodiazepines, antidepressant, and hallucinogens. Although a mass spectrometric approach similar to the one previously described was applied, a new and faster extraction process on micropulverized hair allowed reliable identification and quantification of the target drugs on as little as 2.5 mg of hair samples (Favretto et al., 2012). The combined use of full-scan HRMS and CID offered the possibility of retrospective analysis to detect previously untargeted substances.

D. Biomarkers for Alcohol Abuse

An important goal of forensic toxicology and clinical medicine is to identify appropriate biological markers of ethanol consumption to evaluate harmful drinking and to ascertain alcohol abstinence (Pragst & Balikova, 2006). The determination of direct ethanol metabolites, such as ethyl glucuronide (EtG), ethyl sulfate (EtS), fatty acid ethyl esters (FAEEs), and phosphatidylethanol (PEth), in different biological matrices currently represents the most-valid strategy to provide unbiased evidence of chronic alcohol abuse. Among direct biomarkers, FAEEs and EtG can be detected in a variety of matrices, including hair (Pragst & Balikova, 2006), and allow one to gain information on alcohol consumption for several months. Hair analysis proved very useful to monitor ethanol use/abstinence, workplace testing, driving license reissue/renewal, child custody, divorce proceeding, withdrawal treatment (Politi et al., 2006), and post-mortem or pre-natal alcohol exposure investigation (Bendroth et al., 2008; Pragst & Yegles, 2008), even if the straightforward interpretation of analytical results has been fiercely questioned and warning about possible intra- and inter-individual variability has been raised (Tagliaro et al., 2011).

According to the Society of Hair Testing, analysis of the proximal 3 cm hair segment is recommended in order to avoid partial loss of the analytes. The suggested cut-off values that support a diagnosis of chronic excessive alcohol consumption are 30 pg EtG/mg scalp hair, and 0.5 ng FAEEs/mg scalp hair, respectively; both measured in the 0-3 cm proximal segment (Kintz, 2012b). Figure 3 shows the effectiveness of the 30 pg EtG/mg cut-off value (horizontal line) to discriminate harmful drinkers (red dots) from low-risk drinkers and non-drinkers (yellow and green dots), whereas the cut-off value (vertical line) commonly adopted for an esteemed indirect

biomarker, such as carbohydrate-deficient transferrin (CDT), proved to have reasonable specificity but largely insufficient sensitivity, that led to about 50% false negative results (Pirro et al., 2011a).

In contrast to other forensic applications, a very small number of direct alcohol biomarkers represent for toxicologists the target analytes in hair samples, essentially FAEEs and EtG. Consequently, considerable effort has been devoted to their extraction procedures and instrumental arrangements, in order to obtain continuously improving analytical performances and, above all, minimal LOD and LOQ values. In particular, the refinement of existing methods was addressed to (i) increase the laboratory throughput, with a reduction of sample preparation steps, analysis-time, and costs, (ii) increase the sensitivity and detection capability, so as to accurately quantify minute biomarkers hair concentrations (in the pg/mg range), especially to ascertain alcohol abstinence, (iii) improve the method validation protocols in order to consider all potential sources of bias and uncertainty and assure highly reliable analytical results. Under such circumstances, quite traditional instrumentations, such as triple-quadrupole mass spectrometers in the SRM mode, combined with either GC or HPLC, were almost uniquely utilized, because they assure sensitive and accurate quantification of few pre-determined target analytes.

1. FAEE determination in hair samples

Due to the relatively high volatility and hydrophobicity of FAEEs, HS-SPME-GC/MS is the suggested method-of-choice for the routine determination of ethyl myristate (E14), ethyl palmitate (E16), ethyl stearate (E18), and ethyl oleate (E18:1) in hair extracts (Kintz, 2012b). Among over 20 ethyl esters detected in hair, these four selected analytes were found to best reflect harmful drinking (Pragst et al., 2001). In brief, deuterated internal standards are added to decontaminated hair samples, and subsequently extracted overnight with a heptane-dimethylsulfoxide mixture. The hydrocarbon layer is evaporated and the residue dissolved into a phosphate buffer, on which HS-SPME is executed. The SPME fiber is analyzed with GC/MS in the electron impact (EI) ionization mode under SIM conditions.

Over time, laboratories interested in testing for FAEEs adopted the protocol (Pragst et al., 2001), with small changes for in-house revalidation of the method and to optimize the experimental conditions, to determine E14, E16, E18 and E18:1, with the purpose to better clarify the reliability, limitations, and potential of FAEE biomarkers in hair to ascertain harmful drinking. Later, Pragst and Yegles presented an updated procedure, with detection limits ranging between 0.003-0.01 ng/mg, as determined on 30 mg of hair (Pragst & Yegles, 2008).

Kulaga et al. investigated the potential effect of hair pigmentation on FAEEs incorporation into the keratin matrix, and used, for the first time, hair collected from rats as a model matrix for FAEE production and hair inclusion (Kulaga et al., 2009). As expected for neutral and lipophilic molecules such as FAEEs, they concluded that FAEE concentration in hair does not depend on hair pigment. However, they noted that FAEE hair concentration in rats was much lower than that typically observed in humans, at comparable dosage, but still measurable with the analytical protocol adopted. Gareri et al. used the same analytical method to study the potential bias from use of ethanol-containing hair-care products on FAEE concentration measured on hair of a non-compliant female population (Gareri et al., 2011). From the comparison of FAEE and EtG concentrations in the same hair samples, they concluded that use of hair-care products that contain ethanol (even at low percentage; i.e., less than 10% by volume) might affect FAEE levels in real hair samples, with experimental values from approximately 0.5 to 5.0 ng/mg, to possibly produce false non-compliant outcomes (Gareri et al., 2011). Süsse et al. considered the experimental results from as many as 1872 hair samples from forensic cases (Süsse et al., 2012). They deduced that (i) the use of hair spray might affect (increase) detected FAEEs, but not EtG, hair concentrations; (ii) conversely, bleaching and dying hair treatments might affect (decrease) EtG, but not FAEEs, hair concentrations, whereas (iii) hair gel, hair wax, oil, and grease have apparently no influence on FAEEs and EtG.

Süsse et al. reported a method with detection limits ranging from 0.008 to 0.026 ng/mg, as measured on 20-50 mg hair samples (Süsse et al., 2010). These analytical features allowed the same authors to accurately measure the basic levels of hair FAEEs in strict teetotalers: the results showed that 20% of abstainers (N=242) presented total a FAEE concentration that exceeded the cut-off values of 0.5 or 1.0 ng/mg, as a function of the examined hair length (Süsse et al., 2012). In contrast, Albermann et al. reported total a FAEEs concentration lower than 0.2 ng/mg for 74% of drivers who self-claim as abstinent (N=160), whereas only 9% of these samples showed a FAEE concentration that exceeded 0.5 ng/mg, among which some true-positive cases (Albermann et al., 2011).

Lately, Hastedt et al. updated and revalidated the HS-SPME-GC/MS method originally developed by Pragst et al. (Hastedt et al., 2012; Pragst et al., 2001). Better detection limits were achieved (0.004-0.014 ng/mg), although an expanded time to complete the chromatography cycle was needed (over 30 min) than in the original method.

On the other hand, Caprara et al. and Kulaga et al. described a different analytical strategy to quantify a larger number of FAEEs in neonatal human and animal hair samples, with ethyl heptadecanoate as the internal standard (Caprara et al., 2006; Kulaga et al., 2006). They adopted

solid-phase extraction (SPE) in place of SPME to isolate FAEEs from hair extracts, followed with either GC/MS or GC/MS/MS in the chemical ionization (CI) mode, with isobutene as the ionizing gas. Unlike EI, which typically leads to an extensive, but nearly identical, fragmentation patterns for all FAEEs, CI virtually generates only the protonated molecule ion, which is distinctive for each ethyl ester. MS/MS performed in an ion-trap device clearly adds specificity to the method. In choosing CI rather than EI for the analysis, good detection limits were achieved (<2.4-6.6 pg/mg), only recently outperformed, as reported above. FAEE-analysis profile was also expanded to six, rather than four esters, by adding ethyl laureate (E12), and ethyl palmitoleate (E16:1), and cumulative analysis of all commonly occurring FAEEs in biological matrices was suggested to provide a more effective way to identify neonates with suspected pre-natal exposure to ethanol (Caprara et al., 2006; Kulaga et al., 2006).

By taking advantage of the preceding experiences, Zimmermann and Jackson proposed a globally revisited analytical method to determine five FAEEs (E12, E14, E16, E18, and E18:1) in human hair, and aimed to combine high sample throughput with analytical performances (Zimmermann & Jackson, 2010). Instead of extensive washing procedures and an overnight extraction, followed by a 20 or 40 min-long GC/MS analysis, they proposed a method that required a total analysis-time of less than 1 hour, including a 15-minute sonication-extraction procedure, followed with HS-SPME-GC/MS/MS, performed with a quadrupole ion trap. Positive-CI combined with MS/MS provided high selectivity and detection capability, despite a total chromatography cycle time of only 9 min. The limits of detection ranged from 0.002 to 0.030 ng/mg (Zimmermann & Jackson, 2010). More recently, Politi et al. developed a sensitive (LOQ = 0.01 ng/mg for each FAEE) analytical protocol for FAEE determination in hair, based on the widespread SPE and GC-EI-MS techniques (Politi et al., 2011). These techniques are affordable by any toxicology laboratory - a key feature to make this determination routine. Four ethyl esters (E14, E16, E18, and E18:1) were chromatographically separated in about 16 min. Faster chromatographic analysis is prevented by the need to obtain well-separated peaks and no interferences, because FAEEs share most EI fragment ion signals.

2. EtG determination in hair samples

EtG is a polar substance with a relatively low molecular weight. Due to these attributes, a single-stage of MS analysis is generally insufficient to comply with the low detection limits in the pg/mg range required for its determination in a complex keratinic matrix. Either GC/MS/MS after derivatization or LC-MS/MS are the methods of choice for EtG determination in hair samples; both

are capable to minimize the background interferences and to achieve the optimal detection capability and high specificity, required in toxicological and forensic applications. The limitations of the simple GC-EI-MS approach for EtG determination in hair samples is indirectly demonstrated by a recently developed method, which barely reaches an LOD of 100 pg/mg with as much as 100 mg of hair in the SIM acquisition mode (Álvarez et al., 2009). This LOD value is considerably higher than the established cut-off limit to evidence alcohol use disorders (Kintz, 2012b).

Although the simplicity and progressively increasing performances of ESI make the LC-MS/MS approach prevalent nowadays, several highly-efficient GC/MS/MS methods have been recently developed and updated to fulfill the legal requisites entailed by regulations of many countries. Paul et al. proposed and validated a GC/MS/MS procedure, with EI, triple-quadrupole mass spectrometer, and SRM (Paul et al., 2008). The instrumental analysis followed water extraction, SPE purification, and derivatization with N,O-bis[trimethylsilyl]trifluoroacetamide (BSTFA). An LOD of 5 pg/mg was obtained, and the chromatography cycle was 7.30 min (Paul et al., 2008). A modified protocol was reported by the same group in 2011, in which the SRM transitions for GHB were added to the same analytical protocol, so as to detect EtG and GHB within a single chromatographic procedure. This combination is of importance in the investigation of drug-facilitated crimes, because GHB and alcohol are frequently combined substances used to perpetrate these offences (Paul et al., 2011).

Large-volume injection (LVI) GC-EI-MS/MS was proposed to increase the sensitivity in hair EtG determination. The authors suggested that, upon optimization of streaming volume, purge time, programmable temperature vaporization (PTV) temperature, injection volume, and other experimental parameters, the detection capability can be improved by at least 1–2 orders of magnitude, as compared to the traditional approach (Shi et al., 2010). Even so, they estimated an LOD value of 5 pg/mg, with 20 mg of hair, as in the method previously cited (Paul et al., 2008).

NCI with methane was proposed as an alternative to EI within a GC/MS/MS protocol in order to improve analytical sensitivity (Kharbouche et al., 2009). A high cross-section for electron capture was induced by double derivatization of EtG with perfluoropentanoic anhydride. The choice of two selective SRM transitions allowed high specificity in the analyte identification, despite the fast GC protocol. The estimated LOD value for EtG was 3 pg/mg, with 30 mg hair. This analytical protocol was adopted to analyze rat hair samples, so as to evaluate any potential hair-pigmentation effect on EtG incorporation into the keratin matrix. No statistically significant differences were observed between pigmented and non-pigmented hair (Kharbouche et al., 2010). The same group also evaluated the possible effect of hair-care products on EtG incorporation (Sporkert et al., 2012).

A similar GC-NCI-MS/MS approach was also proposed (Agius et al., 2010), but the final EtG enrichment was performed with HS-SPME, after derivatization with heptafluorobutyric anhydride. HS-SPME injection yielded further clean-up in the headspace extraction and accumulation of the derivatized analyte on the fiber, with a significant decrease of the LOD value: 0.6 pg/mg, with 10 mg hair (Agius et al., 2010). No further improvements were recorded with nonafluorobutyrric anhydride for EtG derivatization instead of heptafluorobutyric anhydride. A similar LOD value (0.7 pg/mg) was also achieved (Kerekes et al., 2009), from 20-30 mg of hair, although NCI was followed by single MS detection, with two monitored ions in the SIM acquisition mode. The EtG concentration was in hair collected from several parts of the body for the same subjects determined and compared (Kerekes et al., 2009).

Even though the GC/MS/MS procedures for EtG determination in hair proved to be accurate and highly sensitive, especially when electron capture was used, most toxicology laboratories currently prefer LC-MS/MS, because the sample-purification steps are easier and no derivatization is needed. These simplifications substantially contribute to reduce the work and time for sample processing - a crucial aspect in laboratories with high sample load.

Although the first LC-MS/MS method dates back to 2002, the real bloom of analytical procedures based on LC separation and ESI started in 2007. ESI is commonly used in the negative ion mode because of the acidic nature of EtG. Among the large variety of proposed methods, different sample-preparation steps, amounts of analyzed hair, and chromatographic conditions were adopted that yielded excellent selectivity and LOD values between 1 and 10 pg/mg. The proposed methods proved highly reliable to support evaluation of harmful drinking (Morini et al., 2009; Marques et al., 2010; Pirro et al., 2011a; Agius et al., 2012); monitor total abstinence (Kronstrand et al., 2012); study hair-treatment effects that possibly lead to partial disappearance of EtG from the keratin matrix (Morini et al., 2010); and evaluate the effect of individual and external parameters on EtG accumulation and persistence in hair, so as to define the boundaries and to exclude conditions for its use as a biomarker of ethanol consumption (Pragst et al., 2010, Süsse et al., 2012; Hastedt et al., 2012; Cabarcos et al., 2012; Pirro et al., 2011b). Almost all these methods made use of triple-quadrupole instruments to monitor SRM transitions between the deprotonated molecule ion of EtG (*m/z* 221) and two fragment ions (*m/z* 75, 85). For EtG determination in hair samples, a detailed list of MS conditions used in the cited articles is presented in Table 1. Conditions for GC and LC methods are reported.

The major critical point of hair EtG determination with LC-ESI-MS/MS methods is the possible occurrence of matrix effects (Matuszewski et al., 2003; Peters & Remane, 2012), especially when a non-polar stationary phase is used for LC separation; i.e., EtG eluted quickly. In any case, matrix

effects need to be carefully evaluated during method development and validation (Lamoureux et al., 2009; Tarcumnicu et al., 2010; Cabarcos et al., 2012). Several authors proposed to modify previously developed analytical protocols in order to improve sample clean-up (essentially with SPE) and consequently reduce ion-suppression and increase the signal-to-noise ratio. Politi et al. reported an extreme case, in which SPE clean-up proved useful to decrease ion suppression and identify EtG traces in three hair segments of a known alcoholic, 27 years after his death (Politi et al., 2008).

Carbon-blend SPE columns, based on porous graphitic material, are suitable for the extraction of EtG from complex matrices such as hair (Lamoureux et al., 2009). Besides, the use of silica HPLC columns, in place of reversed-phase columns, allows one to elute EtG with a mobile phase that contain a high percentage of organic solvent and low percentage of water, to improve ESI efficiency (Lamoureux et al., 2009). An alternative strategy, proposed previously, made use of post-column addition of acetonitrile to the eluate. For the same goal, the use of HILIC coupled to mass spectrometry was proposed and successfully applied for the analysis of EtG in hair (Kintz et al., 2008; Tarcumnicu et al., 2010). Recently, Albermann et al. proposed a modification of their method for EtG determination in hair, aimed to solve occasional chromatographic problems during routine work (Albermann et al., 2012). The new method used an LC column with stationary phase composed of 100% porous graphitic carbon, instead of the reversed-phase LC column previously used (Albermann et al., 2010). However, the new method led to a longer analysis-time and slightly higher LOD value (1.7 pg/mg) than previously reported (1 pg/mg).

E. Antidoping Analysis

In amateur and professional sport, the artificial enhancement of the athletic performance with drugs or forbidden practices is subjected to systematic control. Doping controls in sport events are managed by the World Anti-Doping Agency (WADA), through tests of urine or blood sample collected from athletes in and out of competition. Hair analysis is currently not accepted as an alternative to urine and blood analysis for doping control (WADA, 2012) for several reasons, including its heterogeneous nature, the uncertainty on mechanisms that regulate drug incorporation, and alleged diversity in occurrence and extent of these mechanisms among different ethnic groups (Kintz, 2007a). However, it is evident that hair analysis would be of practical significance in several circumstances, because it provides complementary information with respect to urine and blood testing (Gaillard et al., 2000; Kintz, 2003). In fact, urine and blood analyses detect recent drug intake, but cannot distinguish between chronic use or single exposure, whereas hair analysis can offer this distinction (Kintz, 2007a). Indeed, doping agents are partially excreted in the sweat and

incorporated into growing hair, and remain stable for long periods of time. Thus, hair testing might offer a large detection window to allow long-term detection of prohibited drugs and a retrospective estimation of the intake period. This opportunity appears to be particularly important to detect out-of-competition doping treatments; for example, in the athletes who use anabolic substances during the training period and interrupt their intake long before the start of competition (Kintz et al., 2000).

The need for equal conditions for all athletes and the freedom for them to make use of whatever cosmetic treatment, including complete body shaving, prevented any official use of hair analysis in doping controls. For example, the incorporation rates of prohibited drugs was proved to depend on the hair pigmentation, to possibly lead to unequal conditions in doping control, and consequently ethnic discrimination (Gambelunghe et al., 2007). In addition, hair washing, discoloring, and tinting appear to influence the drug concentration measured in hair, as well as their distribution within the hair longitudinal axis (Kintz, 2003). Nevertheless, the potential of hair analysis for antidoping purposes is so strong that several analytical methods were developed in the past to detect doping agents in hair specimens (Kintz, 2007a).

In the last five years, a limited number of analytical methods was additionally developed, with the main objective to measure anabolic androgenic steroid (AASs) level in human hair with gas or liquid chromatography coupled to high-resolution mass spectrometry or tandem mass spectrometry (Anielski, 2008; Gambelunghe et al., 2007; Deshmukh et al., 2010). A complex challenge was addressed to exploit hair analysis to distinguish between natural production of endogenous steroids (e.g., testosterone) and exogenous uptake of the same steroids (Shen et al, 2009; Pozo et al, 2009, Deshmukh et al. 2012). AASs represent the most frequently detected class of substances in out-of-competition doping testing, because they are widely abused to increase strength and lean body mass, and also lead to reduced recovery periods (Thieme et al., 2000; Pozo et al., 2009; Deshmukh et al. 2010). AASs are also utilized to speed up muscle growth in cattle and horses (Gaillard et al., 1999; Anielski, 2008). AAS detection in hair specimens is more widely accepted in animals than in humans; it still receives a considerable interest in the scientific research, as demonstrated recently (Nielen et al., 2011) in which hormone and veterinary drug screening and, in general, forensic investigations can benefit from the recent developments in desorption electrospray ionization (DESI) mass spectrometry (Takáts et al., 2004). DESI allowed bovine hair analysis with very limited sample preparation: after rapid ultrasonic liquid extraction, a few microliters of supernatant were deposited onto a glass or PTFE surface, and dried, before DESI analysis. Full-scan and MS^3 experiments with a linear ion trap MS were used to detect estradiol benzoate, testosterone cypionate, and testosterone decanoate to demonstrate the general feasibility of rapid screening and

742 detection of anabolic steroid intact esters. Quite high levels (300–800 g/kg) of steroid esters are
743 generally present in hair samples from controlled and illegally treated animals (Nielen et al., 2011).
744 Anielski published a method to detect anabolic steroids and their esters in hair material
745 (Anielski, 2008). After hair extraction (sonication, methanol, 4h, 50°C) and LLE raw purification, a
746 refined sample clean-up procedure was carried out with HPLC. The residue was subsequently
747 derivatized, and the trialkylsilyl steroid derivatives were analyzed with GC/HRMS and GC/MS/MS
748 to yield LOD values between 0.1 and 5.0 pg/mg. The method was successfully applied to real
749 samples to detect testosterone propionate in the hair of treated horses. Unlike a urine specimen, the
750 parent drugs instead of their metabolites are more frequently detected in hair samples.
751 The goal to determine the natural occurrence of anabolic steroids in hair, and their biological
752 variability within selected populations of individuals, led Shen et al. to develop a specific
753 GC/MS/MS method to simultaneously identify testosterone, epitestosterone, androsterone,
754 etiocholanolone, and dehydroepiandrosterone (DHEA) (Shen et al., 2009). After alkaline digestion
755 (NaOH, 1N, 10 min, 90°C), LLE extraction with diethylether was carried out. The extracts were
756 derivatized with a MSTFA/iodotrimethylsilane/dithioerythritol mixture (10:5:5, v/v/w) and
757 analyzed with GC/MS/MS/SRM. The method was fully validated and applied to 80 subjects (39
758 males, 30 females, 11 children). The sensitivity of the method (LODs between 0.1 and 0.2 ng/mg)
759 proved sufficient to determine the physiological concentration ranges for the selected endogenous
760 steroids in the three populations, so as to address the interpretation of non-compliant results from
761 alleged steroid abuse (Shen et al., 2009).
762 An analogous GC/MS/MS method was previously developed for the simultaneous
763 determination of methyltestosterone, nandrolone, boldenone, fluoxymesterone, cocaine, and
764 benzoylecgonine, and was applied to hair samples from seven athletes with reported AAS abuse
765 (Gambelunghe et al., 2007). All anabolic steroids included in the procedure could be detected with a
766 10 pg/mg detection limit. Methyltestosterone, nandrolone, boldenone, and fluoxymesterone were
767 detected in real samples between 12 pg/mg and 37 pg/mg.
768 A specific LC-MS/MS method was developed for the clinical determination of testosterone
769 undecanoate in hair samples (Pozo et al., 2009). After decontamination and incubation in a tris(2-
770 carboxyethyl)phosphine hydrochloride solution (1.5h, 50°C) within an ultrasonic bath, a double
771 LLE with n-pentane was performed. The reconstituted residue was injected into an LC-MS/MS
772 triple-quadrupole mass spectrometer with APCI source. The proposed fragmentation pattern of
773 testosterone undecanoate is shown in Figure 4. The method was applied to hair samples from three
774 patients treated with testosterone undecanoate and led to detected concentrations of 0.4, 1.6 and 8.4
775 pg/mg.

The group of Naughton and coworkers recently published two sensitive LC-MS/MS methods for the detection of various steroids in human hair samples. In the first study, nandrolone and stanozolol were determined in human hair for the first time (Deshmukh et al., 2010). After alkaline digestion (NaOH, 1M, 15 min, 95°C) and LLE with n-pentane, the extracts were injected into a UHPLC system interfaced to a triple-quadrupole mass spectrometer with ESI source. From 20 mg aliquots of hair, LOD values of 0.5 pg/mg for stanozolol and 3.0 pg/mg for nandrolone were obtained. The method was successfully applied to 19 hair samples previously tested positive with ELISA (enzyme-linked immunosorbent assay) screening to reveal seven false-positive results and to confirm one sample positive to nandrolone (14.0 pg/mg) and eleven samples positive to stanozolol (concentration range 5.0÷86.3 pg/mg). The second study used a similar experimental approach to develop a highly sensitive LC-MS/MS method to determine testosterone and epitestosterone in hair samples (Deshmukh et al., 2012). The natural abundance ratio of these steroids (T/E) is significantly modified by any exogenous intake of testosterone. Low LOD values (0.1 pg/mg and 0.25 pg/mg for testosterone and epitestosterone, respectively, from 50 mg of hair) allowed the determination of physiological testosterone and epitestosterone levels on 75 real samples, which ranged from 0.70 to 11.81 pg/mg in males and 0.33 to 6.05 pg/mg in females (testosterone); from 0.63 to 8.27 pg/mg in males and 0.52 to 3.88 pg/mg in females (epitestosterone).

III. FUTURE TRENDS OF HAIR ANALYSIS

A. New Designer Drugs

In the last years, forensic laboratories have been challenged worldwide with the puzzle to detect a variety of new psychoactive substances. These compounds, occasionally called either designer drugs or legal highs, have little or no previous history of medicinal use. Amphetamine derivatives, with backbone molecular structures of piperazine and cathinone, initially represented the main group of designer drugs. Almost at the same time, another wide group of compounds, known as synthetic cannabinoids, appeared on the black market. A very rapid proliferation of new psychoactive substances of these and other classes followed in recent years. Although most of the latest drugs act as central nervous system (CNS) stimulants, their assorted chemical structures range from derivatives of pipradrol, ketamine, phencyclidine, arecoline, aminopropylbenzofuran, ring-

substituted aminoindans, thiophenyl bioisosteres of methamphetamine, as well as compounds structurally related to cocaine (King & Kicman, 2011).

Reference standards for most designer-drugs metabolites are not yet commercially available. That lack of standards poses a serious and longstanding challenge to toxicological laboratories to develop analytical procedures to detect their presence in a variety of biological matrices. Nevertheless, several analytical methods were developed to determine some of these new psychoactive substances and/or their metabolites in either blood or urine (Moran et al., 2011; Grigoryev et al., 2011; Dresen et al., 2011; Teske et al., 2010; Wintermeyer et al., 2010; Beuck et al., 2011; Kraemer et al., 2009; Sobolevsky, Prasolov, & Rodchenkov, 2010). On the other hand, only very few studies have been published to date that describe protocols for the detection of these substances in hair samples.

In a Letter to the Editor, Torrance and Cooper reported the detection of mephedrone in hair samples at 4.2 and 4.7 ng/mg concentration with a ISO/17025 accredited method, but details on the analytical method used and comments or interpretations of results were not included (Torrance & Cooper, 2010). A specific and accurate method for mephedrone detection with GC/MS was proposed (Martin et al., 2012). The study reported that 67 real hair samples were analyzed - 10 were found positive, with mephedrone concentrations from 0.2 to 313.2 ng/mg; 8 of them were below 6 ng/mg. As for amphetamines, it is deduced that mephedrone concentrations in hair are likely in the ng/mg, not pg/mg, at least in the cases of repeated abuse (Martin et al., 2012). Detection of piperazine-like compounds was meticulously described (Barroso et al., 2010b). This study was the first to report the development and validation of an analytical method for the determination of three phenylpiperazines in hair samples. Trimethylsilyl derivatives were determined with GC/MS, and the method was applied to autoptic samples and samples collected from subjects under psychiatric evaluation.

The first multiclass screening for synthetic cannabinoids in hair samples was published recently (Salomone et al., 2012b). In this study, a UHPLC-MS/MS procedure was developed and validated to determine the possible presence of five synthetic cannabinoids (i.e., consistently present in the black market during the period considered: JWH-018, JWH-073, JWH 200, JWH-250, and HU-210) in 179 real hair samples collected from previously proven Cannabis consumers (Figure 5). The fourteen (7.82%) samples found positive to at least one synthetic cannabinoid demonstrated significant diffusion among drug abusers. Concentrations of synthetic cannabinoids ranged from 0.50 to 730 pg/mg. Although the published screening was limited to relatively few compounds, its UHPLC-MS/MS approach makes further method expansion feasible, so as to include new substances that are continuously introduced into the black market.

B. Future Trends Driven by Instrumental Developments

After years of pioneering studies, hair analysis for toxicology applications represents nowadays a reliable and well-established means of forensic investigation. The Society of Hair Testing periodically meets to exchange scientific experiences, establish new protocols, and draw up consensus documents to carefully guide conclusions and legal judgments on the basis of experimental results from hair analysis.

Whereas instrumental sensitivity and overall method specificity have been for years key issues of experimental studies and analytical method developments, the latest instrumentation for sample treatment, chromatographic separation, and mass spectrometric detection substantially fulfills these requirements, and frequently provides LOD and LOQ values largely better (i.e., lower) than the expected drug concentrations in hair. Indeed, various sources of individual variability, including genetic polymorphisms, metabolic disorders, diet, use of cosmetics, and environmental exposure, are likely to represent more-important causes of bias and incorrect reporting than insufficient sensitivity and specificity. For example, the LOD for EtG in hair will soon break the 1 pg/mg limit and will open up a reflection on the possible origins of this alcohol metabolite other than ingestion of alcoholic beverages. Moreover, the chance of external contamination of hair samples and extracts grows with increasing method sensitivity, in turn becoming a major concern during the experimental method validation.

Other issues, basically linked to one another, are becoming increasingly important in forensic hair analysis. These issues are: multiresidue and multiclass potential, high throughput, and costs of analysis. Capability of simultaneous multiclass analysis is essential in broad drug screening of unknown analytes, because in most acute intoxications and post-mortem investigations the ingested substance cannot be anticipated; however, this feature also contributes to dramatically decrease the number of analysis for each sample, with a resultant cost lowering. Modern UHPLC-MS/MS instruments with triple-quadrupole analyzers can already simultaneously detect more than one hundred substances in just a few minutes, and QuEChERS-like procedures to extract hair samples are rapidly spreading within toxicology laboratories. This trend will be probably pursued further in the near future, up to new limits in terms of speed and number of target analytes, so as to manage hundreds of samples per day. Automatically-driven high-resolution MS/MS instruments, sequentially generating data-dependent CID product-ion spectra, will provide true general unknown screening and will allow retrospective inquiry of previously acquired data. Improvements in mass analyzers that will lead to enhanced sensitivity and mass resolution at high scanning rate will

possibly make these instruments preferable to triple quadrupoles for general unknown screening analysis.

High throughput and costs of analysis are strictly connected to one another. Hair analysis is still an expensive determination that prevents its generalized application to workplace and driving license testing, even if it is evident that drivers and, for example, workers who carry fire-arms should not be habitual drug consumers. The analysis costs are due to the labor-intensive hair-sample treatment and the difficulty to test all drugs of abuse at once. Further development of direct MS methods of analysis, such as DESI or MALDI-TOF, combined with simplified sample handling, could possibly provide very fast and cheap drug screening of hair samples in the future. Even if the sensitivity of these instrumental approaches is still low, quite high cut-off values could be accepted whenever the scope of the screening is to single out only the chronic drug consumers. Also, MS-imaging techniques require significant improvement, in terms of hair sample handling and instrumental sensitivity, before generalized application can be found in the chronological assessment of drug intake.

The scientific literature reports no studies on the application of isotope-ratio mass spectrometry (IRMS) to hair analysis to discriminate the natural vs. exogenic origin of substances present therein. The substances of interest might include doping agents, such as steroids, as well as a common rape drug; i.e., gamma-hydroxybutyric acid. The reasons for this lack can be found again in the limited sensitivity of the technique, the restricted sample availability, the complexity of its purification, and that specificity provided by mass selection is lost in the combustion process. The chance of using IRMS to detect the illegal administration of drugs over extended periods of time, as it might occur for androgenic steroids, will possibly push some scientific efforts in this direction in the near future.

In general, the recent major improvements of MS and chromatographic instrumentation have driven forensic toxicology toward previously inconceivable results. Among these studies, it is impressive to note that hair analysis can nowadays provide evidence of a single administration of drugs. The continuous enhancement of MS sensitivity progressively reduces the amount of hair sample needed for the chemical analysis, and extends the chance to investigate newborn children, to detect pre-natal drugs exposure. The scientific goal to gain toxicological information from a single hair has already started, in a worthy competition with forensic genetics. Further unexpected developments of MS instruments are likely to have immediate impact also in the investigations of forensic toxicology, and in particular in hair analysis.

IV. ABBREVIATIONS

All abbreviations and acronyms used in this article are listed in Table 2.

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TABLE 1. Comparison of MS conditions for EtG determination in hair samples

Authors, year	Analytical Technique	Mass Analyzer	*m/z* Ions (acquisition mode)		Derivatization
Álvarez et al., 2009	GC-EI-MS	single quadrupole	261; 160; 405	(SIM)	BSTFA
Paul et al., 2008 Paul et al., 2011	GC-EI-MS/MS	triple quadrupole	261→ 143	(SRM)	BSTFA
Shi et al., 2010	GC-EI-MS/MS	triple quadrupole	261→ 143 261→ 73	(SRM)	BSTFA
Kharbouche et al., 2009 Kharbouche et al., 2010 Sporkert et al., 2012	GC-NCI-MS/MS	triple quadrupole	347 → 163 347 → 119	(SRM)	PFPA
Agius et al., 2010	GC-NCI-MS/MS	triple quadrupole	596 → 427 596 → 288	(SRM)	HFBA
Kerekes et al., 2009	GC-NCI-MS	single quadrupole	496; 349	(SIM)	PFPA
Morini et al., 2009 Morini et al., 2010 Politi et al., 2008	LC-ESI-MS/MS	triple quadrupole	221→ 221 221 → 85 221 → 75	(SRM)	-
Pirro et al., 2011a Pirro et al., 2011b Kronstrand et al., 2012 Pragst et al., 2010 Hastedt et al., 2012 Lamoreux et al., 2009 Tarcumnicu et al., 2010 Kintz et al., 2008	LC-ESI-MS/MS	triple quadrupole	221 → 85 221 → 75	(SRM)	-
Süsse et al., 2010 Süsse et al., 2012	LC-ESI-MS/MS	triple quadrupole	221 → 75 221 → 57 221 → 55	(SRM)	-
Cabarcos et al., 2012	LC-ESI-MS/MS	ion trap	221 → 203 221 → 85 221 → 75	(SRM)	-
Albermann et al., 2010 Albermann et al., 2012	LC-ESI-MS/MS	triple quadrupole	221 → 113 221 → 85 221 → 75	(SRM)	-

TABLE 2. Abbreviations and acronyms used in this article.

6-MAM	6-monoacetylmorphine
AASs	Anabolic Androgenic Steroids
AP	Amphetamine
APCI	Atmospheric Pressure Chemical Ionization
BSTFA	N,O-bis[trimethylsilyl]trifluoroacetamide
BZE	Benzoylecgonine
CBN	Cannabinol
CDB	Cannabidiol
CDT	Carbohydrate-Deficient Transferrin
CI	Chemical Ionization
CID	Collision-Induced Dissociation
CNS	Central Nervous System
CZE	Capillary Zone Electrophoresis
DESI	Desorption Ionization Mass Spectrometry
DFC	Drug Facilitated Crime
DFSA	Drug Facilitated Sexual Assault
DHEA	Dehydroepiandrosterone
E12	Ethyl laureate
E14	Ethyl myristate
E16	Ethyl palmitate
E16:1	Ethyl palmitoleate
E18	Ethyl stearate
E18:1	Ethyl oleate
EI	Electron Impact
ELISA	Enzyme-Linked ImmunoSorbent Assay
EME	Ecgonine Methyl Ester
ESI	Electrospray Ionization
EtG	Ethyl Glucuronide
EtS	Ethyl Sulfate
FAEE	Fatty Acid Ethyl Esters
FTICR	Fourier Transform Ion Cyclotron Resonance
GC	Gas Chromatography
HFBA	Heptafluorobutyric Anhydride
HILIC	Hydrophilic Interaction Liquid Chromatography
HPLC	High Pressure Liquid Chromatography
HRMS	High Resolution Mass Spectrometry
HS-SPME	Head Space Solid-phase MicroEstraction
IMS	Imaging Mass Spectrometry
IRMS	Isotope-Ratio Mass Spectrometry
LC	Liquid Chromatography
LLE	Liquid-Liquid Extraction
LOD	Limit of Detection
LOQ	Limit of Quantification

TABLE 2. (Continued)

LVI	Large Volume Injection
LTP	Low Temperature Plasma
MALDI	Matrix-Assisted Laser Desorption Ionization
MBDB	N-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine
MBTFA	N-methyl-bis trifluoroacetamide
MDA	3,4-methylenedioxyamphetamine
MDEA	3,4-methylenedioxyethamphetamine
MDMA	3,4-methylenedioxymethamphetamine
MiAMi	Micropulverized Extraction Aqueous Acetylation Microextraction
MIP	Molecularly Imprinted Polimers
MISPE	Molecularly Imprinted Solid-Phase MicroExtraction
MS	Mass Spectrometry
MSTFA	N-methyl-N-trimethylsilyltrifluoroacetamide
MTBSTFA	N-methyl-N-(tert-butyldimethyl)trifluoroacetamide
NCI	Negative Chemical Ionization
PEth	Phosphatidylethanol
PTV	Programmable Temperature Vaporization
QqQ	Triple quadrupole mass spectrometer
QqQLIT	Hybrid triple quadrupole linear ion trap mass spectrometer
QuEChERS	Quick Easy Cheap Effective Rugged Safe
SIM	Selected Ion Monitoring
SOHT	Society of Hair Testing
SOHT	Society of Hair Testing
SPE	Solid-phase Extraction
SRM	Selected Reaction Monitoring
THC	Δ^9-tetrahydrocannabinol
TMCS	Trimethylchlorosilane
TOF	Time of Flight
UHPLC	Ultra High Performance Liquid Chromatography
WADA	World Anti-Doping Agency

FIGURE CAPTIONS

FIGURE 1. MALDI-MS analytical workflow for single hair analysis. The whole process from sample preparation to SRM relative quantitation and MS/MS and MS^3 confirmatory analyses takes approximately 2 h, including the analysis of multiple samples (Porta et al., 2011; reproduced with permission, American Chemical Society, copyright 2011).

FIGURE 2. GC×GC contour plot (TIC) obtained for a hair sample. (1) Cotinine; (2) meconin; (3) mesocain; (4) tropacaine; 5.EDDP; (6) phenazocine; (7) hydroxycotinine-TBDMS; (8) methadone; (9) quaalude; (10) norcocaine; (11) cocaine; (12) cocaethylene; (13) codeine; (14) diazepam; (15) benzoylecgonine-TBDMS; (16) acetylcodeine; (17) cinnamoylcocaine; (18) diacetylmorphine; (19) codeine-TBDMS; (20) morphine-TBDMS; (21) 6-MAM-TBDMS; (22) Papaverine; (23) morphine-di-TBDMS (Guthery et al., 2010; reproduced with permission, Elsevier, copyright 2010).

FIGURE 3. Concentration of head hair EtG (pg/mg) versus %CDT for 175 patients clinically classified as non-drinkers (A, green dots, N=65), social drinkers (S, yellow dots, N=51), and active heavy drinkers (H, red dots, N=59). The data are reported on a logarithmic scale. Horizontal and vertical lines divide the plane into four quadrants (I-IV) that identify the number of true and false positive (or negative) results in each category: For heavy drinkers (red dots): I. True-positive results; II. false-negative results for CDT only; III. false-negative results; IV. false-negative results for head hair EtG only (Pirro et al., 2011; redrawn with permission, Springer, copyright 2011).

FIGURE 4. Proposed fragmentation pattern for testosterone undecanoate (Pozo et al., 2009; reproduced with permission, John Wiley and Sons, copyright 2009).

FIGURE 5. SRM chromatogram of a blank hair sample fortified with the target compounds: (1) JWH-200, (2) JWH-250, (3) JWH-073, (4) CBD, (5) JWH-018, (6) CBN, (7) HU-210, (8) THC, (ISTD) JWH-018-*d9* (Salomone et al., 2012; reproduced with permission, John Wiley and Sons, copyright 2012).